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Mechanisms of Natural Gene Therapy in Dystrophic Epidermolysis Bullosa

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Revertant mosaicism has been reported in several inherited diseases, including the genetic skin fragility disorder epidermolysis bullosa (EB). Here, we describe the largest cohort of seven patients with revertant mosaicism and dystrophic EB (DEB), associated with mutations in the *COL7A1* gene, and determine the underlying molecular mechanisms. We show that revertant mosaicism occurs both in autosomal dominantly and recessively inherited DEB. We found that null mutations resulting in complete loss of collagen VII and severe disease, as well as missense or splice-site mutations associated with some preserved collagen VII function and a milder phenotype, were corrected by revertant mosaicism. The mutation, subtype, and severity of the disease are thus not decisive for the presence of revertant mosaicism. Although collagen VII is synthesized and secreted by both keratinocytes and fibroblasts, evidence for reversion was only found in keratinocytes. The reversion mechanisms included back mutations/mitotic recombinations in 70% of the cases and second-site mutations affecting splicing in 30%. We conclude that revertant mosaicism is more common than previously assumed in patients with DEB, and our findings will have implications for future therapeutic strategies using the patient's naturally corrected cells as a source for cell-based therapies.

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INTRODUCTION

Inherited epidermolysis bullosa (EB) is the prototypic mechanobullous disease, characterized by the development of blisters following apparently minor trauma or traction of the skin (Gedde-Dahl, 1971). It encompasses four major forms: simplex, junctional, dystrophic EB, and Kindler syndrome, and is caused by mutations in 18 different genes (Fine *et al.*, 2008; Bruckner-Tuderman and Has, 2012; Intong and Murrell, 2012). Dystrophic epidermolysis bullosa (DEB, OMIM #226600) is characterized by a broad spectrum of clinical severity from very mildly affected patients with only nail dystrophy to the severe generalized form with widespread blisters, massive scarring, and mitten deformities. Mutations in the gene for collagen VII (Col7), *COL7A1,* are the cause of all the DEB subtypes (Chung and Uitto, 2010). The disease can be inherited in a dominant (DDEB) or in a recessive manner (RDEB) (Kern *et al.*, 2009). All forms of DEB, but especially the severe generalized RDEB (RDEB-sev gen) subtype, present as a painful, chronic disease with a high, unmet medical need and a strong impact on the patient's quality of life. So far, the treatment is only symptomatic, and novel therapeutic strategies are urgently needed.

Somatic reversion leading to a clinically healthy phenotype, so-called revertant mosaicism (RM), has been reported in hematological conditions and several genodermatoses (Davis and Candotti, 2010). The first case of molecularly confirmed RM and genetic skin disease was described in 1997 in a 28-year-old patient with junctional EB (JEB)-non-Herlitz. RM has since been observed in all the main EB types (Pasmooij *et al.*, 2005, 2007; Jonkman and Pasmooij, 2012). Here, we describe the largest cohort so far of seven patients with different DEB phenotypes and RM, and clarify the reversion mechanisms.

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Abbreviations: Col7, type VII collagen; DDEB, dominant dystrophic epidermolysis bullosa; DEB, dystrophic epidermolysis bullosa; EB, epidermolysis bullosa; JEB, junctional epidermolysis bullosa; RDEB, recessive dystrophic epidermolysis bullosa; RDEB-o, recessive dystrophic epidermolysis bullosa, generalized other; RDEB-sev gen, recessive dystrophic epidermolysis bullosa, severe generalized; RM, revertant mosaicism; SNP, single nucleotide polymorphism

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RESULTS

In all seven DEB patients, healthy skin patches were found amidst blistered and atrophic/scarred skin. The patients never had blisters on these areas. The origin, clinical features, and mutation constellation of the patients are summarized in Table 1. Patient 1 had DDEB-generalized (DDEB-gen) and patient 2 had mild RDEB-generalized (RDEB-gen), whereas patients 3–7 had the severest type, RDEB-sev gen.

Inherited mutations

All the patients with RDEB were compound heterozygous for *COL7A1* mutations, except for patient 5 who was homozygous. Patients 2, 5, and 6 had the recurrent splice-site mutation c.425A>G, which generates out-of-frame transcripts with a premature termination codon and is therefore, functionally, a null mutation. The c.425A>G mutation is identified in 13% of the alleles of DEB patients with Central European origin (Csikos *et al.*, 2005). Patients 3 and 4 carried the recurrent frameshift mutation c.6527dupC; p.Gly2177Trpfs*113 in a heterozygous state. This mutation is present in 46% of the RDEB alleles in the Spanish population (Escamez *et al.*, 2010) (Table 1).

Clinical features and immunofluorescence staining

All patients were specifically asked whether they had unaffected skin areas, that is, areas where no blisters occurred even after mechanical friction. The areas indicated were located on the extremities, except for patient 7 who had a healthy appearing patch on the back, next to large wounds and residual scarring. The patches had normal pigmentation and skin texture, and hair was present in some patches.

Skin specimens from the healthy appearing areas, together with specimens of affected skin from the same patient, were subjected to immunofluorescence staining with antibodies to Col7. The signal was absent or reduced in the affected skin of all patients, whereas it was restored to 20–100% in the patches of clinically unaffected skin, suggestive of reversion. The staining intensity of the revertant patches was comparable to that of control skin in patients 1 (Figure 1), 2 (Figure 2), 4 (Supplementary Figure S1 online), and 6 (Supplementary Figure S3 online), whereas the staining intensity was restored to a lower extent in patients 3 (Figure 3), 5 (Supplementary Figure S2 online), and 7 (Supplementary Figure S4 online).

Patient 1 suffered from DDEB-gen with moderate blistering, which was restricted to the extremities and rarely on the oral mucosa. As a second diagnosis, from the age of 16 years, the patient had developed an atopic dermatitis with erythematous, itchy skin, IgE levels of $>5{,}000$ IU ml^{-1}, and a predisposition to allergies. We observed several healthy appearing patches next to scarred, atrophic, and erythematous skin (Figure 1a).

Patient 2 had mild RDEB-gen with blisters and subsequent scarring, almost exclusively on the extremities. On his left lower leg, patches of unaffected skin were observed, surrounded by wounds or scarred skin (Figure 2a). The patient could not recall how long these patches had been there, but he said they were not expanding. Because Col7 staining of the affected skin was only slightly reduced, it was a challenge to confirm the RM by IF staining.

Patients 3–7 had the characteristic clinical features of RDEB-sev gen with widespread blistering, extensive scarring of skin and mucous membranes, and development of mitten deformities on hands and feet. Patients 5 and 6 had a history of squamous cell carcinomas. A biopsy was obtained from the right hand of patient 6 (Supplementary Figure S3a online). Col7 staining was strongly reduced in this specimen and detachment at the dermal-epidermal junction zone was visible. However, a short stretch of basal keratinocytes stained brightly for Col7, providing evidence for RM in this small skin specimen, that is, ''micro-mosaicism'' (Supplementary Figure S3b online).

Molecular mechanisms of revertant mosaicism

Following immunofluorescence staining, the molecular mechanisms of *in vivo* reversion in the keratinocytes were assessed. Laser dissection microscopy (LDM) was used to collect keratinocytes from areas with positive (revertant) and reduced or negative Col7 staining (mutant). Subsequently, DNA was amplified and the regions containing the *COL7A1*

Table 1. Patients with dystrophic epidermolysis bullosa and revertant mosaicism in this study

Patients	EB subtype	Age, origin	1st *COL7A1* mutation cDNA	Exon	Consequence	2nd *COL7A1* mutation cDNA	Exon	Consequence
#1	DDEB	23, German	c.6127G>A	73	p.Gly2043Arg	N/A	N/A	N/A
#2	RDEB-gen	63, German	c.425A>G	3	Altered splicing→PTC	c.8206G>A	110	p.Glu2736Lys
#3	RDEB-sev gen	21, Spanish	c.2142A>G	16	Splice-site mutation resulting in out-of-frame transcript lacking the last 29 base pairs of exon 16	c.6527dupC	80	p.Gly2177Trpfs*113
#4	RDEB-sev gen	22, Spanish	c.884delG	7	PTC	c.6527dupC	80	p.Gly2177Trpfs*113
#5	RDEB-sev gen	37, German	c.425A>G	3	Altered splicing→PTC	c.425A>G	3	Altered splicing → PTC
#6	RDEB-sev gen	17, German	c.425A>G	3	Altered splicing→PTC	c.1837C>T	14	p.Arg613*
#7	RDEB-sev gen	12, German	c.4894C>T	51	p.Arg1632*	c.6176A>G	73	p.Glu2059Gly

Abbreviations: DDEB, dominant dystrophic epidermolysis bullosa; N/A, not applicable; PTC, premature termination codon; RDEB-o, recessive dystrophic epidermolysis bullosa, generalized other; RDEB-sev gen, recessive dystrophic epidermolysis bullosa, severe generalized.

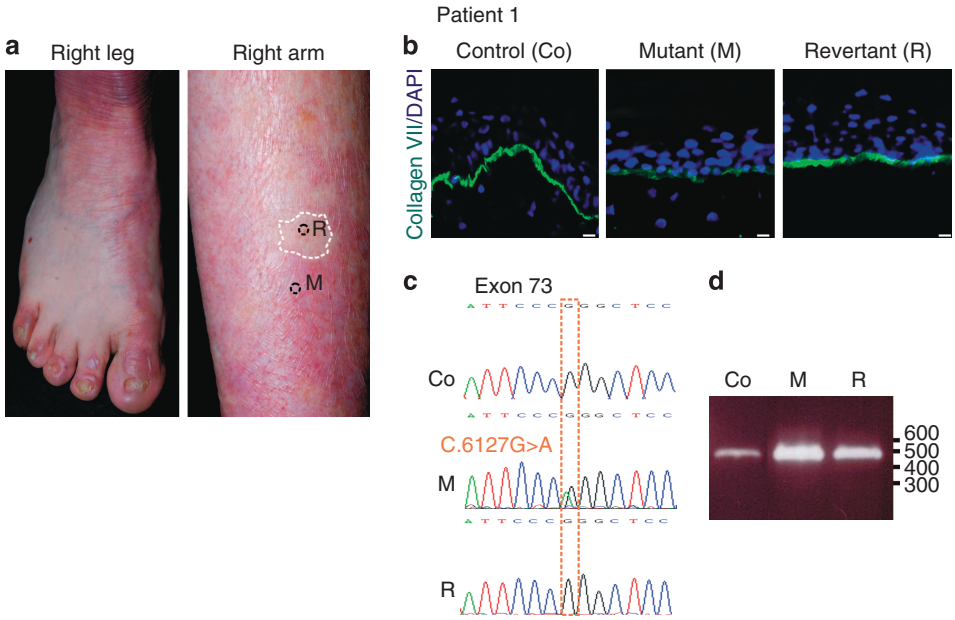

Figure 1. Clinical features and identification of reversion mechanism in patient 1. (**a**) She had blisters, scarred and atrophic skin mostly on the lower legs and feet, as well as dystrophic toenails. However, on the right arm, several healthy appearing patches were identified next to scarred, atrophic, erythematous skin. A biopsy was taken from one of the revertant patches (R) and from neighboring scarred skin (M). (**b**) Col7 was reduced in the mutant skin (M), but showed a strong positive staining in the revertant skin (R). Bar = 20 μm. (**c**) Partial sequence of *COL7A1* exon 73 revealed the mutation c.6127G > A in affected keratinocytes and the lack of it in keratinocytes from unaffected skin. (**d**) Reverse transcriptase–PCR (RT-PCR) spanning exons 70–76 showed no effects on splicing. Bar = 50 μm.

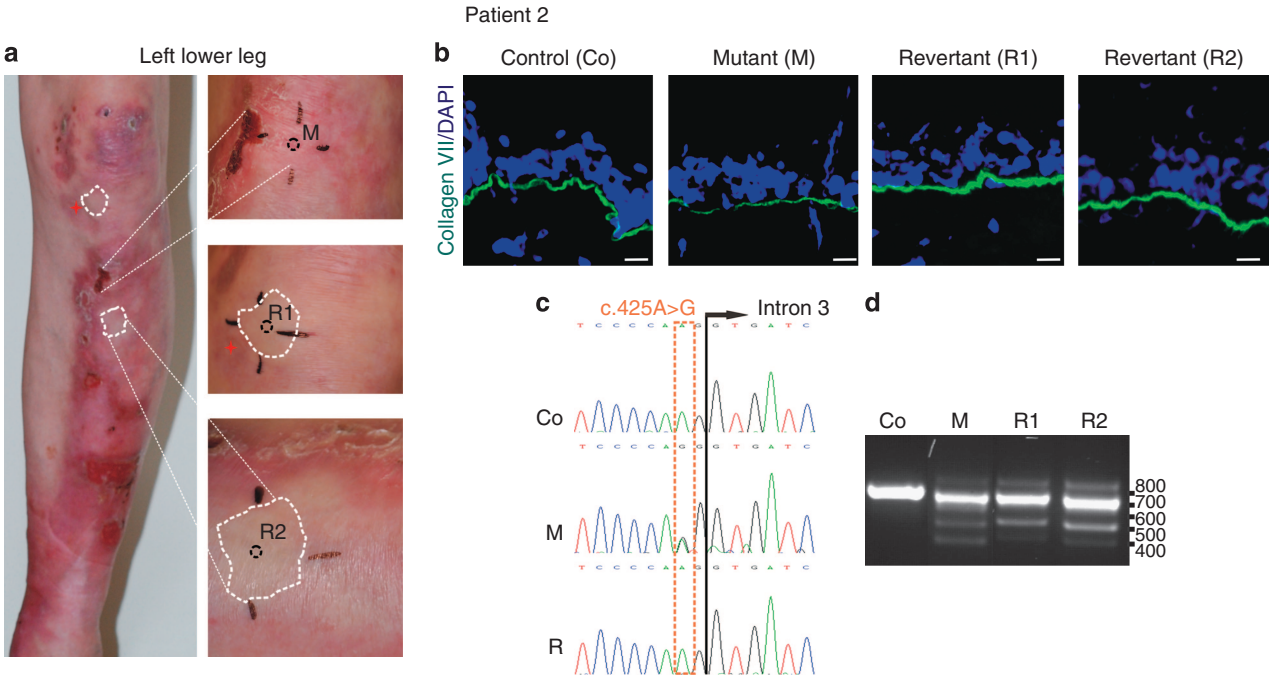

Figure 2. Clinical features and identification of reversion mechanism in patient 2. (**a**) Biopsies were taken from two healthy skin spots (R1 and R2) on the left lower leg and affected neighboring skin (M). (**b**) Col7 was slightly reduced in the mutant skin (M), whereas it was strongly positive in the revertant skin (R1 and R2). Bar = 50 μm. (**c**) Partial sequence of *COL7A1* exon and intron 3 showed the mutation c.425A > G in affected keratinocytes, whereas it was absent in keratinocytes from unaffected skin. (**d**) Reverse transcriptase–PCR (RT-PCR) with primers spanning exon 2–5 revealed the same transcripts in all samples. However, the normal transcript appeared to be enhanced in the samples from the revertant areas on the electrophoresis gel. Bar = 50 μm.

mutations were sequenced. In all cases, the dermal fibroblasts and patients' lymphocytes were also analyzed. To determine the mechanism on a transcriptional level, RNA was isolated from sections of revertant and mutant skin, and reverse transcriptase–PCR was performed with primers spanning the region surrounding the mutations (Table 2).

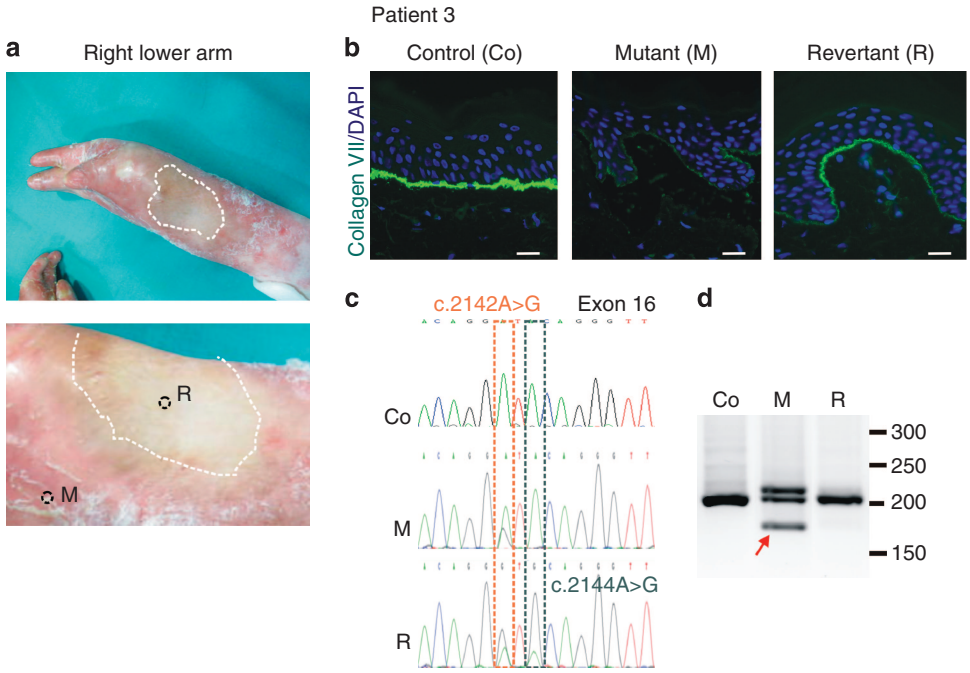

Figure 3. Clinical features and identification of reversion mechanism in patient 3. (**a**) A healthy skin patch on the dorsal side of the right hand. A biopsy was taken from this area (R) and from neighboring affected skin (M). (**b**) Skin of a control individual (Co), and from mutant (M) and revertant (R) areas of patient 3 were stained with Col7 antibody 2Q633. The Col7 expression in the mutant skin was strongly reduced, whereas in the unaffected, revertant skin it was restored, although reduced compared with the control. Bar = 50 μm. (**c**) Partial sequence of *COL7A1* exon 16 in keratinocytes collected from affected skin (M) showed the mutation c.2142A>G, whereas in revertant keratinocytes (R) the additional mutation c.2144A>G was found. (**d**) The effect of the second-site mutation on RNA level was studied by reverse transcriptase–PCR (RT-PCR) with primers spanning exons 15–17. The inherited c.2142A>G mutation resulted in an out-of-frame transcript (lane M) lacking the last 29 nucleotides of exon 16 (red arrow). The larger transcript was a heteroduplex of the wild-type transcript from the c.6527dupC allele, and the transcript lacking the 29 nucleotides. The somatic second-site mutation c.2144A>G in combination with c.2142A>G again resulted in the use of the wild-type splice-site (lane R). Bar = 50 μm.

In patient 1, the dominant mutation was absent. Likewise, in patients 2, 4, and 7, the correction resulted in the absence of one of the recessive mutations in the revertant patches, which suggests a back mutation or mitotic recombination as an underlying repair mechanism. In patient 2, sufficient material was available to discriminate between the correction mechanism of a true back mutation or deletion/mitotic recombination by investigating the flanking introns and exons for single nucleotide polymorphisms. In lymphocyte DNA, the next heterozygous single nucleotide polymorphism was found in exon 21: c. 2945A>G; p.Pro939Pro. This polymorphism was in a homozygous state in the revertant keratinocytes of both unaffected skin patches, pointing to mitotic recombination as the reversion mechanism.

A second-site mutation was identified in patients 3 (Figure 3c–d) and 5 (Supplementary Figure S2c–d online), next to the original splice-site mutation. In patient 5, the additional mutation c.426+3G>A was predicted (Alamut, Rouan, France v2.3) to restore the normal splice site that was disrupted by the germline c.425A>G mutation. In line, RNA analysis showed the expression of the normally spliced transcript at much higher levels (~70% of the analyzed transcripts) than in the mutant skin (~16% of the analyzed transcripts).

In patient 3, the inherited c.2142A>G mutation created an alternative splice site in exon 16 resulting in an out-of-frame transcript lacking the last 29 nucleotides of exon 16. In the unaffected skin biopsy, the additional heterozygous sequence variant c.2144A>G increased the expression levels of the normal splice product, as was also predicted by splice-site prediction software (Alamut). It also introduced a substitution p.Tyr715Cys, which was apparently at least partly functional. This tyrosine is conserved in several species and, according to the prediction program Polyphen-2 (v2.2.2, http://genetics.bwh.harvard.edu/pph/), the substitution likely represents a pathogenic variant. This could explain why the Col7 staining of the revertant stretch, together with the null mutation on the other allele, was not fully restored.

In patient 6, both mutations found in his lymphocytes were present in all LDM-isolated DNA samples, as was the case in the cDNA samples (Supplementary Figure S3c online). No additional mutations were detected in the DNA of the revertant keratinocytes in the regions analyzed. These results suggest that mitotic recombination is the repair mechanism resulting in the presence of both mutations on one allele, and a second allele without mutations. Long-range reverse transcriptase–PCR, spanning exons 2–15, to verify this reversion mechanism could not be performed due to lack of

Table 2. Skin biopsy sites and molecular findings

Patients	Clinical phenotype	Clinical features of revertant skin area					Collagen VII expression in immunofluorescence mapping		Reversion mechanism on genomic DNA level	Reversion mechanism on mRNA level
		Size	Location	Pigmentation	Hair	Observation of patient	% Of revertant cells in the biopsy	Intensity compared with control skin		
#1	Moderate blistering, mostly on extremities. Second diagnosis: atopic dermatitis	3 × 3 cm	Right arm	Normal	Present	No remarks	>50%	Comparable	Back mutation/mitotic recombination: Heterozygous mutation c.6127C>A, p.Gly2043Arg absent	Heterozygous mutation c.6127C>A, p.Gly2043Arg present in less than 10% of the allelic levels
#2	Blisters and scarring on lower legs, arms, and elbows	3 × 3 cm	Left lower leg (R1)	Normal	Absent	The revertant area did not expand	>80%	Comparable	Mitotic recombination: Heterozygous mutation c.425A>G absent and loss of heterozygosity of the neighboring SNP c. 2945A>G; p.Pro939Pro (in both revertant patches)	Identification of normal-spliced transcript, which was not found in the mutant skin
		3 × 3 cm	Left lower leg (R2)	Normal	Absent					
#3	Typical features of RDEB-sev gen	7 × 3 cm	Dorsal side of the right hand	Normal	Absent	Can carry luggage	>50%	Reduced	Second-site mutation: Additional heterozygous sequence variant, c.2144A>G	Identification of normal-spliced transcript with the substitution p.Tyr715Cys
#4	Typical features of RDEB-sev gen	7 × 4 cm	Right lower arm	Normal	Present	Can wear a bracelet. Obvious since 14 years of age	100%	Comparable	Back mutation/mitotic recombination: Heterozygous mutation c.884delG absent	Heterozygous mutation c.884delG absent
#5	Typical features of RDEB-sev gen, squamous cell carcinomas	4 × 4 cm	Lateral side of the lower leg	Normal	Absent	The skin on his whole left lower leg had always been less prone to mechanically induced blisters	80%	Slightly reduced	Second-site mutation: Additional heterozygous sequence variant, c.426+3G>A	Identification of normal-spliced transcript, which was not found in the mutant skin
#6	Typical features of RDEB-sev gen, squamous cell carcinomas	N/A	Right hand during a hand surgery procedure to treat mitten deformities	N/A	N/A	N/A	20%	Comparable	Mitotic recombination: Both mutations found in the patient lymphocytes present, no additional mutations	Both mutations present, no additional mutations
#7	Typical features of RDEB-sev gen. Second diagnosis: atopic dermatitis	10 × 5 cm	Back (R1)	Normal	Present	Photographic documentation showed a similar size over a period of 3 years	80%	Reduced	Back mutation/mitotic recombination: Heterozygous mutation c.6176A>G, p.Glu2059Gly absent (in both revertant skin areas)	Heterozygous mutation c.6176A>G, p.Glu2059Gly absent (in both revertant skin areas)
		5 × 3 cm	Outer side of the right lower leg (R2)	Normal	Present	Obvious since birth				

Abbreviations: N/A, not available; RDEB-sev gen, recessive dystrophic epidermolysis bullosa, severe generalized; SNP, single nucleotide polymorphism.

material. Hence, no molecular proof of the reversion mechanism could be identified in this case and the presence of RM was based only on clinical and immunofluorescence findings.

Functional test for defining areas with RM

In our search for RM in patients with DEB, we recognized that not all the healthy appearing skin areas were revertant. In three additional patients, biopsies from skin patches that appeared normal (patients 8–10, Supplementary Figure S5 online) did not show restored expression of Col7. In these cases, the areas had preserved hair and skin texture, indicating that clinical features alone are insufficient to define whether an area is revertant. Therefore, simple functional tests are required to verify the RM and avoid an invasive skin biopsy. We propose two easy-to-perform tests: the rub test and skin tape stripping. In the rub test only the tip of a retracted ballpoint pen is required. With a quick movement, the tip of the ballpoint is pushed over the skin for a distance of 2–4 mm. The friction is enough to evoke a blister in generalized subtypes of DEB, but not in revertant skin, thereby distinguishing between them (Figure 4). Another approach is skin tape stripping, where an adhesive plaster is placed on the skin area suspected to be revertant and pulled off. If the skin detaches, reversion is excluded (Gostynski *et al.*, 2009).

DISCUSSION

We have described mechanisms of revertant mosaicism in a cohort of seven unrelated patients with different DEB subtypes. The reversion mechanisms are back mutations/mitotic recombinations that result in the absence of a mutation on one allele in 5/7 cases (71%), and second-site mutations that affect splicing in 2/7 cases (29%). The fact that 6/7 patients were compound heterozygous for *COL7A1* mutations shows that mitotic recombination could be the correction mechanism. In agreement with our results, this mechanism of reversion was described earlier in one DEB patient (Almaani *et al.*, 2010), whereas in two other RDEB-sev gen patients, who were homozygous for *COL7A1* null mutations, the reversion mechanism was a second-site mutation (Pasmooij *et al.*, 2010; van den Akker *et al.*, 2012). On the basis of these data, we postulate that in RDEB patients who are compound heterozygous for *COL7A1* mutations, the reversion preferably occurs through back mutations/mitotic recombinations. To differentiate between these two reversion mechanisms, heterozygous single nucleotide polymorphisms surrounding the corrected mutation need to be investigated, such as performed in patient 2 where loss of heterozygosity was reported; this pointed to mitotic recombination as the mechanism of repair. In homozygous patients, mitotic recombination will not result in correction of the inherited mutation, and other mechanisms such as second-site mutations will be observed. In contrast, in the skin fragility disorder Kindler syndrome (Kiritsi *et al.*, 2012), which is caused by recessive mutations in *FERMT1*, only back mutations and mitotic recombinations have been reported so far as the correction mechanism, and no second-site mutations leading to a slightly aberrant kindlin protein have been identified. In the dominantly inherited genetic disorders, ichthyosis with confetti (Choate *et al.*, 2010) and dyskeratosis congenita (Jongmans *et al.*, 2012), only mitotic recombinations have been reported as the correction mechanism, which is similar to the correction mechanism observed in our dominant DEB patient 1. In such patients, as in compound heterozygous recessive patients, mitotic recombination therefore seems to be the preferable mechanism of repair.

Notably, in our DEB patients, the revertant areas had bizarre shapes and could increase to a size of 50 cm^2, which is in contrast to the round revertant patches reaching up to 15 cm^2 described in patients with Kindler syndrome or ichthyosis with confetti. The persistence of revertant clones indicates that the reversion event occurs in epidermal stem cells. As epidermal stem cell units are estimated to populate an area of ~0.25–0.5 mm^2 in human skin (Ghazizadeh and Taichman, 2005), the large size of these areas demonstrates that the revertant cells have a transient selection advantage, as at some point the revertant patches stop growing larger. It cannot be excluded, however, that large reverted areas may represent a confluence of multiple overlapping or neighboring revertant events. Notably, although Col7 is synthesized and secreted by both keratinocytes and fibroblasts, we only found evidence for reversion in keratinocytes. A possible explanation could be the higher turnover of the keratinocytes in combination with a selection advantage, thereby increasing the chance of a correction event occurring and outgrowth of these revertant cells.

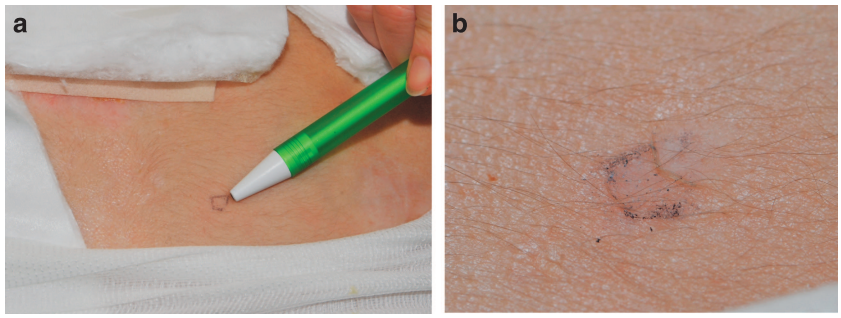

Figure 4. A functional rub test with ballpoint pen to test for revertant skin in a dystrophic epidermolysis bullosa (DEB) patient. (**a**) The round tip of a retracted ballpoint is pushed over the normal appearing skin patch suspected of being revertant mosaicism (RM). This resulted in a blister (**b**), showing that the skin was fragile and the functionality was not restored.

We identified RM in all DEB-generalized subtypes, irrespective of inheritance mode and disease severity. However, it is a challenge to identify RM by immunofluorescence staining in patients with residual Col7 expression associated with a mild phenotype. We found that preserved skin texture and the absence of atrophy were crucial factors in recognizing revertant skin in DEB. In contrast to patients with JEB due to *COL17A1* mutations, hyperpigmentation and presence of hair were not indicative (Pasmooij *et al.*, 2012). Asymmetry was also an important indicator: the presence of an unaffected patch on one side, with the opposite side being strongly affected suggests RM.

It is worth mentioning that three out of seven patients carried the *COL7A1* mutation c.425A>G in a homozygous or heterozygous state; it was this mutation that was corrected in two of these patients by a second-site mutation and mitotic recombination. As the c.425A>G mutation is a common founder mutation of central European origin, we expect more patients with reversion of this c.425A>G mutation to be identified.

Our findings on RM have therapeutic consequences for DEB patients. Revertant keratinocytes isolated from these areas could be cultured into skin grafts and used as an autologous skin transplant on affected skin or mucosal areas. First treatment approaches have already been performed in patients with JEB (Gostynski *et al.*, 2009), and show that the grafting procedure is easy to perform and well tolerated by the patients. However, isolation and culturing of the revertant cells must be optimized before large sheets of cells become available. Another approach could take advantage of induced pluripotent stem cells, as recent reports have stated that mouse and human induced pluripotent stem cells can be differentiated into keratinocytes (Itoh *et al.*, 2011; Tolar *et al.*, 2011; Uitto, 2011). Generation of induced pluripotent stem cells from revertant skin provides an essentially unlimited number of patient-specific cells for grafting or systemic hematopoietic cell transplantation (Uitto, 2011; Tolar *et al.*, 2013).

MATERIALS AND METHODS

Human tissues

After written informed consent, EDTA-blood samples and skin biopsies were obtained from all patients. At least two 4-mm punch biopsies were taken: one from affected and one from unaffected skin. The study was conducted according to the Declaration of Helsinki.

Immunomorphological analysis of the skin

The immunofluorescence mapping of skin cryosections was performed as described before (Kiritsi et al., 2011). Primary antibodies were the monoclonal LH7.2, which recognizes the non-helical carboxy-terminal region of Col7 (Millipore, Schwalbach, Germany), the polyclonal anti-Col7 antibody #234192 (Calbiochem, Bad Soden, Germany), and the C-terminus-specific mAb 2Q633 (US Biologicals, Swampscott, MA). The Alexa Fluor 488–conjugated goat anti-rabbit and anti-mouse IgG antibodies (Invitrogen, Karlsruhe, Germany) were used as secondary antibodies. Nuclei were stained with DAPI (Millipore, Temecula, CA).

Mutation detection

Mutation detection on DNA extracted from EDTA-blood using the Qiagen kit (Qiagen, Hilden, Germany) was performed as described before (Kern *et al.*, 2006). All 118 exons and adjacent junctions of the *COL7A1* gene were amplified and subsequently sequenced. DNA sequences were compared with the reference sequence from National Center for Biotechnology Information (Genbank number NG_007065.1) using the Mutation SurveyorTM DNA variant analysis software (version 2.61 Softgenetics, State College, PA). The mutations were verified by sequencing in both directions and from an independent PCR reaction.

DNA and RNA extraction from skin sections

For DNA recovery by LDM, skin cryosections of 4–5 μm were mounted on 1.0-mm PEN membrane–covered slides (Zeiss, Göttingen, Germany). Keratinocytes or fibroblasts from positively or negatively Col7-stained areas were dissected using the Laser Robot Microbeam System (P.A.L.M. Microlaser Technology AG) or Leica (Leica Microsystems Nussloch GmBH, Nussloch, Germany) Laser Microdissection system 6500, and directly collected in caps of 0.5-ml thin-wall reaction tubes (Zeiss). Approximately 200 keratinocytes microdissected from the epidermis or fibroblasts from the dermal part were collected. A 30-μl mix consisting of PCR buffer, water, and proteinase K (Qiagen) was added. During digestion by proteinase K, the tubes remained for 60 minutes at 55 °C; subsequent heating to 98 °C for 15 minutes inactivated the proteinase K. The final aliquots were used for nested PCR.

For patients 3 and 4, RNA isolation and cDNA synthesis were performed as described in the study by Pasmooij *et al.* (2010). For RNA isolation in patients 1, 2, and 5–7, 10 sections of 10-μm thickness were extracted with the Qiagen FFPE RNA kit (Qiagen) according to the manufacturer's protocol. Reverse transcription was performed using the Advantage RT-for-PCR Kit (Clontech) with 0.3 μg of total RNA, using oligo dT primers. Primers for the regions of interest were designed with Primer3.

Identification of mutations in LDM samples

For detection of mutations in LDM-isolated DNA, nested PCR was used. For the second PCR, 1–2.5 μl of the first PCR product and nested primers were used. PCR cycling conditions were 5 minutes at 94 °C, followed by 35 cycles at 94 °C for 45 seconds, 60 °C for 45 seconds, and 72 °C for 1 minute with a final extension at 72 °C for 10 minute. Water, instead of DNA, was used as a negative control. All PCRs were repeated with templates from at least three separate DNA isolations obtained by LDM, and all products were sequenced in both directions.

Cloning

RNA from cryosections of mutant and revertant skin patches was used as a template for the reverse transcriptase–PCR. When more than two PCR products were observed on the electrophoresis gel, the amplicons were subcloned into the TOPO TA-cloning vector (Invitrogen). For each reaction, 15–20 clones were sequenced using the M13-RV primer, disclosing the different transcripts that arise.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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